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CHARACTERIZATION OF GERMINATION INHIBITORS IN SEED EXTRACTS OF FOUR SOUTH AFRICAN SPECIES OF PROTEACEAE*

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Abstract

Water extracts of the seed coat and embryo of *Protea compacta, P. barbigera, Leucospermum cordifolium* and *Leucadendron daphnoides* were separated chromatographically in *n*-butanol:ammonia:water and ethyl acetate:ammonia. The lettuce seed bioassays used for the detection of germination inhibitors in the extracts showed that the major inhibitor present had an R_f value similar to coumarin. A second inhibitor, present in some extracts, had an R_f value similar to ABA. Ether extracts and ethyl acetate extracts separated in *n*-butanol:ammonia: water confirmed the findings obtained from water extracts. The lettuce seed bioassays used for the detection of germination promotors gave no indication of their presence in ether extracts.

A UV spectrophotometric analysis of the ethyl acetate extracts of *Protea compacta* showed that the major inhibitor, with the same R_f value as coumarin, had the same UV absorbance peak as the coumarin standard. The inhibitor with the same R_f value as ABA was shown to have a different absorbance peak to the ABA standard.

Uittreksel

KARAKTERISERING VAN ONTKIEMINGSTREMSTOWWE IN DIE EKSTRAKTE VAN SAAD VAN VIER SUID AFRIKAANSE PROTEACEAE.

Waterekstrakte van die saadhuid en embrio van *Protea compacta, P. barbigera, Leucospermum cordifolium* en *Leucadendron daphnoides* is met behulp van *n*-butanol:ammonia:water en etielasetaat:ammonia chromatografies geskei. Die slaaisaadbiotoetse wat gebruik is, toon dat die hoofstremstof teenwoordig in dié ekstrakte 'n R_f-waarde het wat ooreenstem met die van koemarien. 'n Tweede stremstof, wat in sekere ekstrakte aanwesig is, het 'n R_f-waarde in ooreenstemming met dié van absissiensuuir (ABS). Eterekstrakte en etielasetaatekstrakte wat met behulp van *n*-butanol:ammonia:water geskei is, bevestig die bevindings wat verkry is deur middel van waterekstrahering. Die slaaisaadbiotoetse wat aangewend is om 'n aanduiding van enige ontkiemingsnelstowwe te gee, was negatief.

Spectrofotometriese analise van die etielasetaatekstraksies van *Protea compacta* toon dat die hoofstremstof, naamlik dié met dieselfde R_f-waarde as koemarien, ook dieselfde UV absorpsiepiek as die koemarien-standaard het. Die stremstof met dieselfde R_f-waarde as ABS het egter 'n absorpsiepiek gehad wat verskil met dié van die ABS-standaard.

Introduction

In an earlier investigation (Brown and Van Staden, 1971) it was shown that the aqueous extracts (diffusates) of the seed coats and embryos of *Protea compacta, P. barbigera, Leucospermum cordifolium* and *Leucadendron daph-*

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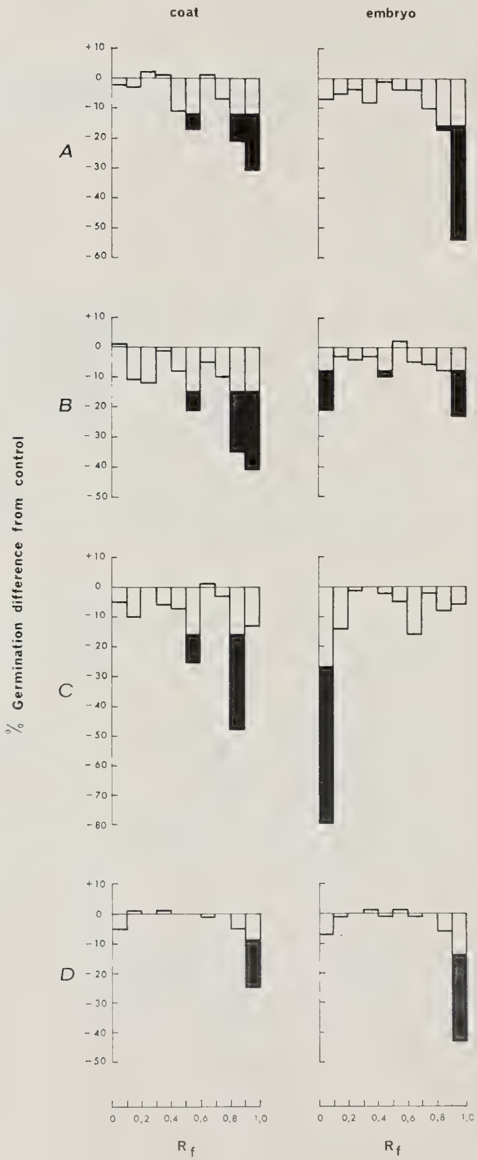
coat
embryo
A
B
C
D
% Germination difference from control
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
0,2
0,4
0,6
0,8
1,0
R_f

FIG. 1.

noides inhibited either seed germination or subsequent root growth of seedlings of lettuce and water cress. It was shown that this inhibition could not be accounted for on the basis of osmotic pressure alone. Chromatographic separation of the seed coat and embryo extracts of all species showed a band of inhibition corresponding to R_f values of 0,9—1,0. These results were compared with the inhibitory effects of two widely occurring germination inhibitors, viz. coumarin and ABA. Coumarin was reported by Evenari (1949; 1961) and Wareing (1965) to be of wide occurrence in plant tissue and to be one of the most potent of the naturally occurring germination inhibitors. Since its discovery by Okhuma, Smith, Lyon and Addicott (1963), ABA has been found to be associated with the dormancy of seed of a number of species, e.g. peach (Lipe and Crane, 1966), rice (Dey and Sircar, 1968), and apple (Rudnicki, 1969). Previous work indicated that, after separation in *iso*-propanol:ammonia:water, the coumarin and ABA standards could not be distinguished from one another and from the seed extracts when compared on the basis of the inhibition produced in the bioassays.

In this paper further chromatographic techniques were used in an attempt to characterize the inhibitors occurring in the seed extracts. Special attention was given to a comparison of the properties of these inhibitors with those of coumarin and ABA to determine whether the latter inhibitors were present in the seeds.

MATERIAL AND METHODS

Seed used in all experiments was purchased from the Department of Forestry, Pretoria.

Techniques for extraction of seed.

(*a*) *Water extract.* The technique for obtaining water extracts of the seed coats and embryos (including the cotyledons) was basically the same as described earlier (Brown and Van Staden, 1971). The only modification was that the leachate was concentrated to dryness under reduced pressure at 40°C. The residue was taken up in one ml distilled water and 500 µl of this was immediately strip-loaded on to a chromatogram.

(*b*) *Ether extract.* The method of extraction was based on that of Eagles and Wareing (1964) and Irving and Lanphear (1968). Fifteen grams of seed was separated into coats and embryos; each was homogenized in a blendor at room temperature in 80% aqueous methanol. The fractions were kept overnight in a refrigerator and then filtered through Whatman No. 42 filter paper. The

FIG. 1.

A comparison of the effects of water extracts of seed coats and embryos on germination of "Grand Rapids" lettuce seed in light.

A = *Protea compacta;* B = *P. barbigera;* C = *Leucospermum cordifolium;*
D = *Leucadendron daphnoides*

The solvent was *n*-butanol:ammonia:water. The shaded areas represent differences significant at 1% level.

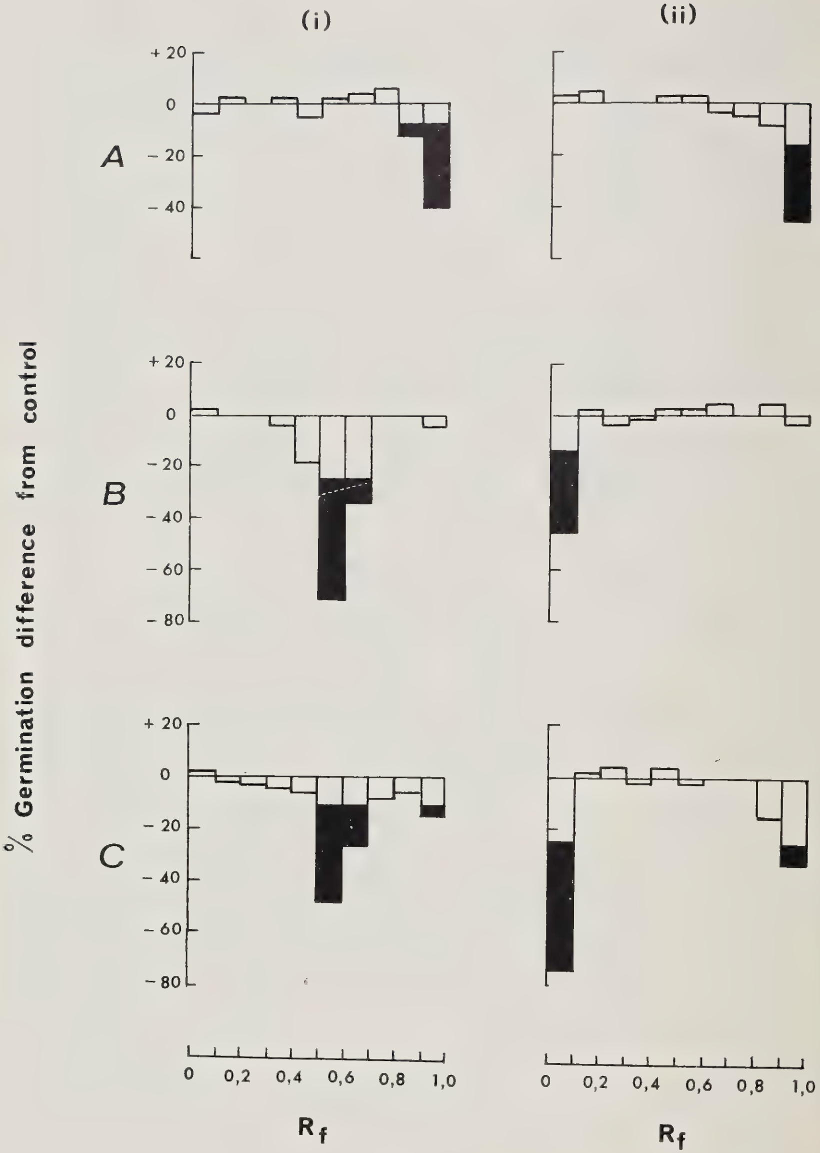
(i)
(ii)
A
B
C
+ 20
0
− 20
− 40
− 60
− 80
% Germination difference from control
0
0,2
0,4
0,6
0,8
1,0
R_f

FIG. 2.

fractions were concentrated at 30°C under reduced pressure in order to remove the methanol. The aqueous fraction was acidified to pH 2,0 with 5% H_2SO_4 and then extracted six times with 50 ml ether. The ether fraction was reduced to dryness under reduced pressure at 40°C. The residue was dissolved in six ml dry ether, three ml of which was loaded immediately on each of two chromatograms. One chromatogram was used in a lettuce seed germination bioassay in the light and the other for a similar bioassay, but in the dark.

(*c*) *Ethyl acetate extract.* The extraction technique used was similar to that used by Davis, Heinz and Addicott (1968) for the extraction of ABA. Fifteen grams seed was separated into coats and embryos. Each was homogenized in 100 ml 80% acetone, kept in a refrigerator overnight and then filtered through Whatman No. 42 filter paper. The extracts were then evaporated to an aqueous residue, acidified to pH 2 with HCl and extracted twice with equal amounts of ethyl acetate. The acidic ethyl acetate was combined and extracted twice with equal amounts 5% $NaHCO_3$. The pH of the combined sodium bicarbonate fractions was adjusted to two and it was extracted twice with equal amounts of ethyl acetate. After being reduced to dryness the residue was taken up in 4 ml ethyl acetate and immediately strip-loaded on to chromatograms.

Chromatography

All extracts were separated at room temperature using descending paper chromatography. The concentrated extracts and the standards were strip-loaded on Whatmans No. 1 chromatography paper. The chromatograms were equilibrated for four hours prior to development. After the solvent front had travelled about 40 cm, the chromatograms were dried and cut into ten equal transverse strips. The biological activity of each strip was determined in the lettuce seed germination bioassay.

The water extracts were sparated in two solvent systems viz. (i) *n*-butanol: ammonia:water (100:3:18 v/v), and (ii) the organic phase of ethyl acetate: 2N ammonia (1:1 v/v).

Bioassays

When only the inhibitor activity of extracts was investigated the bioassay was done in the light. When both inhibitor and promotor activity was investigated the bioassay was carried out in the dark. All experiments were repeated at least twice.

FIG. 2.

A comparison of the effects of ABA and coumarin on the germination of "Grand Rapids" lettuce seed in light. The solvents were (i) *n*-butanol:ammonia:water and (ii) ethylacetate: ammonia.

A = Coumarin B = ABA C = Coumarin + ABA

The shaded areas represent differences significant at 1% level.

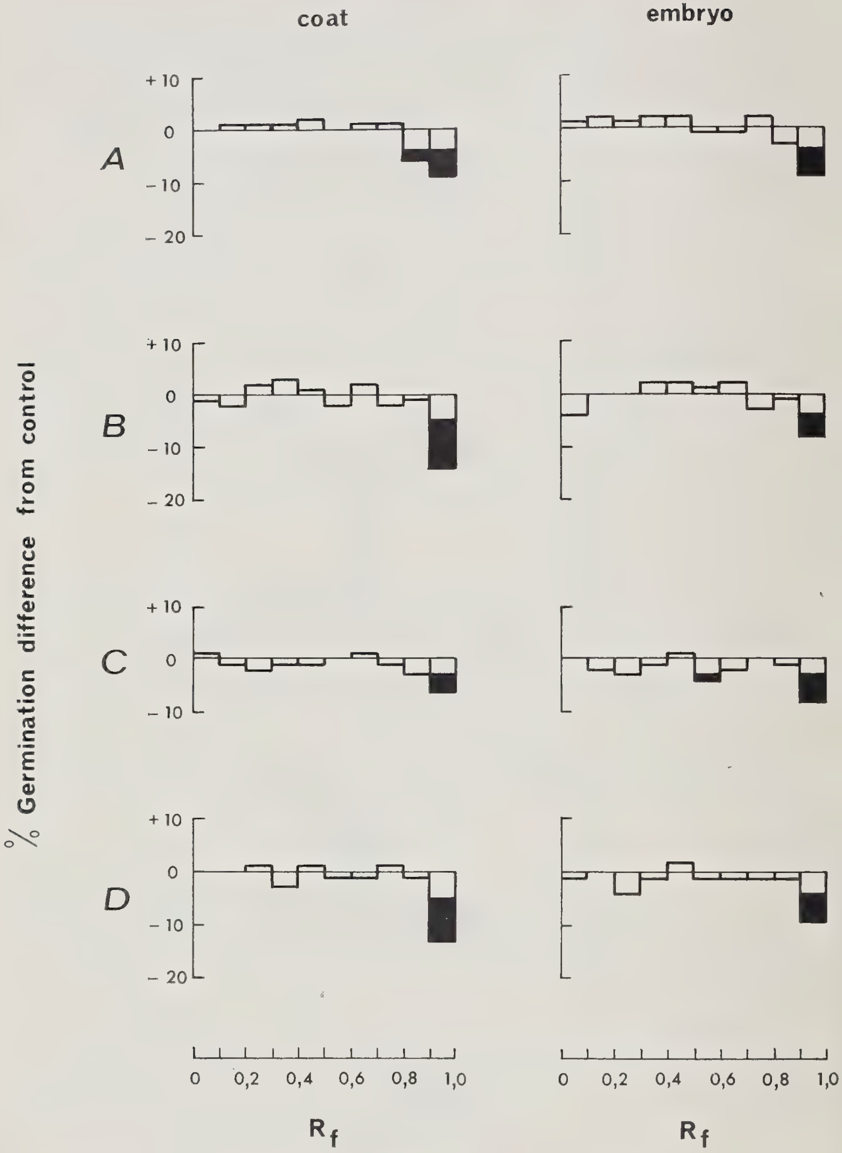
coat
embryo
% Germination difference from control
A
B
C
D
+ 10
0
– 10
– 20
0
0,2
0,4
0,6
0,8
1,0
R_f

FIG. 3

(*a*) *Light germination of lettuce seed.* The bioassay used was based on the techniques of Sankhla and Sankhla (1968). Each strip from the chromatograms was placed in a petri dish, on one sheet of Whatman No. 1 filter paper and three ml distilled water was added. One hundred seeds of lettuce (var. Grand Rapids) were then scattered on the paper in each petri dish and the dishes were placed in the dark for two hours. The lettuce seeds were then exposed to cool white fluorescent light, with an intensity of 3,0 × 10 lumen/m^2, for 30 min. After illumination, the seeds were germinated in the dark in a growth cabinet maintained at 26°C. The percentage germination was recorded after 48 hours.

(*b*) *Dark germination of lettuce seed.* Essentially the same procedure as in (*a*) was followed. The only difference was that after the lettuce seeds were scattered on the filter paper in each petri dish, the dishes were immediately placed in the dark in a growth cabinet at 26°C. The percentage germination was recorded after 48 hours.

Chemicals.

(RS)-abscisic acid (ABA) was obtained through the courtesy of R. J. Reynolds Tobacco Company, North Carolina, U.S.A. The gibberellic acid (GA) used was the potassium salt of GA_3 purchased from Calbiochem, Los Angeles, California. Coumarin was obtained from L. Light and Company, Bucks, England.

RESULTS AND DISCUSSION

(i) *Chromatographic separation of water extracts and bioassay for the detection of germination inhibitors.*

Water extracts were separated in *n*-butanol:ammonia:water as, in this solvent system, abscisic acid separated out in the region of R_f 0,6 (Davison, 1965; Bowen and Hoad, 1968) and coumarin in the region of R_f 0,9 (Swain, 1953). (See Fig. 2.) Results of the bioassay after the separation of water extracts in *n*-butanol:ammonia:water are given in Figure 1. Some difficulty was experienced in obtaining an efficient separation of the embryo extracts of *Leucospermum cordifolium* and *Protea barbigera* in this solvent system. The significant band of inhibition at R_f 0,1 is regarded as being due to the poor separation of the extracts rather than indicating the presence of a specific inhibiting substance. The extracts of the other species all separated out well and, together with the embryo extract of *Protea barbigera*, gave bands of inhibition corresponding either to R_f 0,9 or 1,0 or spread over both values.

FIG. 3.

A comparison of the effects of water extracts of seed coats and embryos on the germination of "Grand Rapids" lettuce seed in light.

A = *Protea compacta;* B = *P. barbigera;* C = *Leucospermum cordifolium;* D = *Leucadendron daphnoides*

The solvent was ethyl acetate:ammonia. The shaded areas represent differences significant at 1% level.

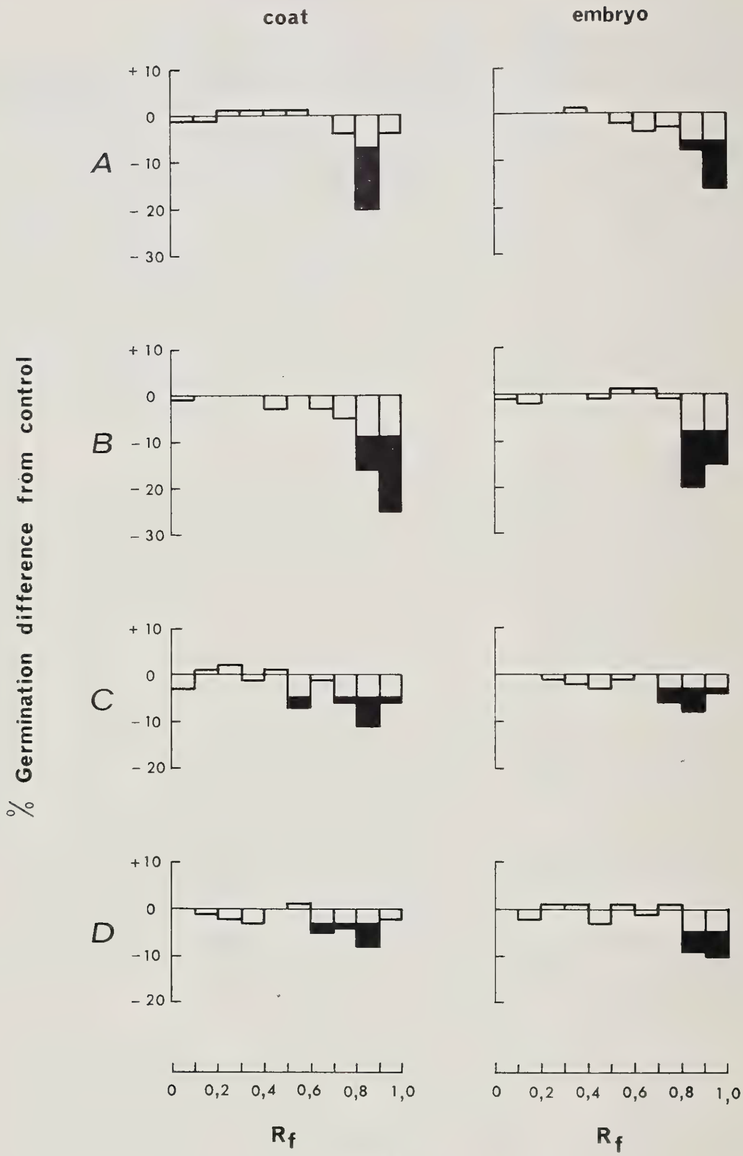
coat
embryo
+ 10
0
- 10
- 20
- 30
A
B
C
D
% Germination difference from control
0
0,2
0,4
0,6
0,8
1,0
Rf

FIG. 4.

Seed coat extracts of *Protea compacta; P. barbigera*, and *Leucospermum cordifolium* showed a second band of inhibition corresponding to R_f 0,6 and the embryo extract of *Protea barbigera* a second band at R_f 0,5.

These results indicated that the major inhibitor of germination in the bioassay was a compound or compounds which separated out in a similar way to coumarin. They also indicated that the second inhibitor, which was present in some of the extracts, separated out to a position on the chromatogram broadly corresponding to the position of ABA.

In a third experiment water extracts were separated in ethyl acetate:2N ammonia. In this solvent system ABA separated out only very slowly and gave a band of inhibition at R_f 0,1. The coumarin standard alone and mixed with ABA gave inhibition corresponding to R_f 1,0 (See Fig. 2). Figure 3 shows that all the seed extracts gave a band of inhibition corresponding to R_f 1,0, with inhibition spreading over R_f 0,9 and 1,0 in the case of the seed coat extract of *Protea compacta*. The embryo extract of *Leucospermum cordifolium* also showed a zone of inhibition at R_f 0,7. These results tended further to suggest that the major inhibitor present in the seed extracts behaved in a manner similar to coumarin.

(ii) *Chromatographic separation of ether extracts and bioassay for the detection of germination promotors and inhibitors.*

A problem encountered in chromatographing the water extracts of seed was that the large number of water soluble substances present appeared to interfere with the efficient separation of the constituents of the extracts. In some cases this was partially overcome by loading smaller amounts of extract on any one strip of the chromatogram and increasing the number of strips. The separate strips were then all combined in the bioassays. A more satisfactory solution to the problem was to use different techniques for extracting the seeds. The extraction of seeds using ether was shown by Eagles and Wareing (1964) and Irving and Lanphear (1968) to be suitable for the extraction of growth inhibitors and promotors. Seeds of all species were extracted using ether and the extracts were separated in *n*-butanol:ammonia:water. Results of the bioassay for the detection of inhibitors are shown in Figure 4 and results for the standards are shown in Figure 2. After separation, all the seed extracts and the coumarin standard showed bands of inhibition corresponding to R_f 0,9—1,0. In addition, the seed coat extracts of *Leucospermum cordifolium* showed a band of inhibition at R_f 0,6 which corresponded with the ABA standard.

FIG. 4.

A comparison of the effects of ether extracts of seed coats and embryo on germination of "Grand Rapids" lettuce seed in light.

A = *Protea compacta;* B = *P. barbigera;* C = *Leucospermum cordifolium;* D = *Leucadendron daphnoides*

The solvent was *n*-butanol:ammonia:water. Shaded areas represent differences significant at 1% level.

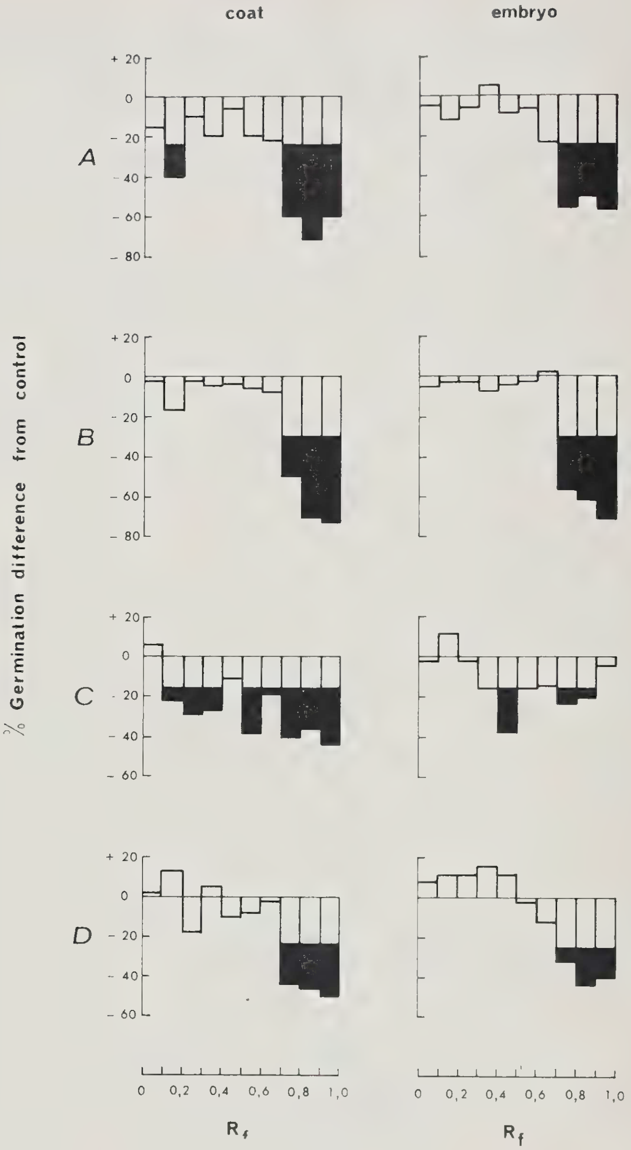
coat
embryo
+ 20
0
- 20
- 40
- 60
- 80
A
B
C
D
% Germination difference from control
0
0,2
0,4
0,6
0,8
1,0
Rf
Rf

FIG. 5

Results of the bioassay (in the dark) for the detection of inhibitors and promotors in the ether extracts are shown in Fig. 5. The results for the ABA and coumarin standards were the same as those shown in Figure 2. The gibberellic acid standard (not shown) gave significant promotion of germination at R_f 0,3. All the seed extracts gave a band of inhibition in the region of R_f 0,8—1,0, which corresponded to that shown by the coumarin standard. In *Leucospermum cordifolium* the seed coat extract showed inhibition at R_f 0,6—0,7 and the embryo extract at R_f 0,5. The ABA standard gave inhibition at similar R_f values (0,5—0,6). Additional bands of inhibition were shown by *Leucospermum cordifolium* coat extracts at R_f 0,2—0,4 and *Protea compacta* coat extracts at R_f 0,2. No evidence of a promotor was obtained in any of the seed extracts. Bioassay results of the ether extracts suggested again that the major inhibitor present had properties similar to coumarin. One or more inhibitors with properties broadly similar to those of ABA were also present in some extracts.

(iii) *Chromatographic separation of ethyl acetate extracts and bioassay for the detection of germination inhibitors.*

As has been mentioned previously and can be seen in Fig. 1, a number of the water extracts gave inhibition in the bioassay in the region of R_f 0,5, a position which closely resembled that of ABA. In order to determine whether ABA was in fact present, an extraction technique used by Davis, Heinz and Addicott (1968) specifically for ABA, was used. Seeds were extracted using ethyl acetate and the extracts separated in *n*-butanol:ammonia:water. Results of the bioassay are given in Figure 6. Results for the standards are the same as in Fig. 2. With the exception of *Leucadendron daphnoides* embryo extract, the seed extracts of all species gave inhibition at R_f 0,9 or 1,0 as also shown by the coumarin standard.

It was interesting to note that although this extraction technique was not the most suitable for extracting coumarin (as the latter is not very soluble in acetone or in ethyl acetate) a substance with properties similar to coumarin was extracted in sufficient quantity to show inhibition in the bioassay.

The seed coat extracts of *Leucospermum cordifolium* and *Leucadendron daphnoides* were the only two extracts to show significant (1%) inhibition in the region of R_f 0,5—0,6 which broadly corresponds with the ABA control. These results indicate that ABA may either be present in extremely small concentrations, or absent.

FIG. 5.

A comparison of the effects of ether extracts of seed coats and embryos on germination of "Grand Rapids" lettuce seed in darkness.

A = *Protea compacta;* B = *P. barbigera;* C = *Leucospermum cordifolium;* D = *Leucadendron daphnoides*

The solvent was *n*-butanol:ammonia:water. Shaded areas represent differences significant at 1% level.

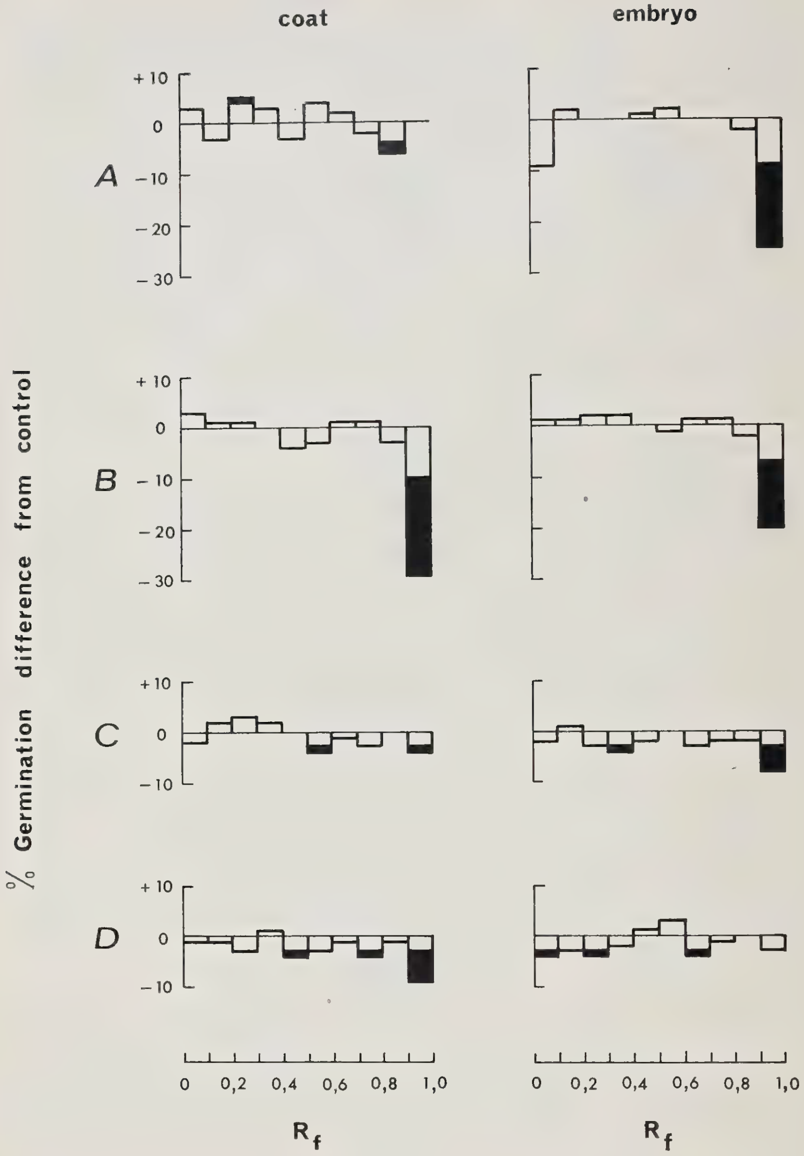
coat
embryo
+10
0
−10
−20
−30
A
B
C
D
% Germination difference from control
0
0,2
0,4
0,6
0,8
1,0
Rf

FIG. 6.

In an attempt to eliminate the concentration factor, six times the weight of seed normally used for extraction was extracted, using ethyl acetate. As seed supplies were limited, only *Protea compacta* was investigated. The extracts were separated in *n*-butanol: ammonia: water, the chromatogram cut into strips and each strip was eluted for 24 hours in 80% methanol. The pattern of absorption of each fraction of the seed coat and embryo extracts under UV was then analysed in a Zeiss DMR 21 Spectrophotometer.

These results (See Fig. 7) show the absorption spectra of coumarin and ABA standards, together with the chromatographed fractions of seed coat and embryo extracts. ABA shows peak absorption at 242 nm and coumarin at 272 nm. The inhibitor in the seed coat and embryo extracts which was shown up in the bioassay at R_f 1,0 (See Fig. 8), shows a peak similar to coumarin at 272 nm. The inhibitor present in the seed coat which separated out at R_f 0,5—0,6 (Fig. 8), has peak absorption at 278 nm and can thus be seen not to be ABA.

In order to further characterize the inhibiting compound which had properties similar to coumarin, the seed extracts of all species were chromatographed in three different solvent systems and the chromatograms were then treated with a number of spray reagents. Each reagent gave a characteristic colour reaction in the presence of coumarin (Swain 1953). The average R_f value for the inhibiting compound in *iso*-propanol: ammonia: water was 0,91 (coumarin control R_f 0,92). In *n*-butanol: ammonia: water the R_f was 0,92 (coumarin control $R_f = 0,92$) and in ethyl acetate ammonia R_f was 0,93 (coumarin control $R_f = 0,94$). The compound which appeared at these R_f values showed up with a yellow/green fluorescence when irradiated with UV after spraying with 2N NaOH. No fluorescence was obtained without pretreatment with NaOH. The active spot also reacted with aqueous 1% potassium permanganate giving a yellow colour. These reactions indicate that the compound is coumarin-like, being in accord with the behaviour described for coumarin by Swain (1953).

The overwhelming evidence is thus that the major inhibitor in the species studied is coumarin-like in its properties. A number of other inhibitors have also been shown to be present. However, no evidence for the presence of ABA in detectable quantities in the seed of *Protea compacta* was found. This does not necessarily preclude ABA from being present in the seed of other species.

FIG. 6.

A comparison of the effects of ethyl acetate extracts of seed coats and embryos on the germination of "Grand Rapids" lettuce seed in light.

A = *Protea compacta;* B = *P. barbigera;* C = *Leucospermum cordifolium;* D = *Leucadendron daphnoides*

The solvent was *n*-butanol:ammonia:water. Shaded areas represent differences significant at 1% level.

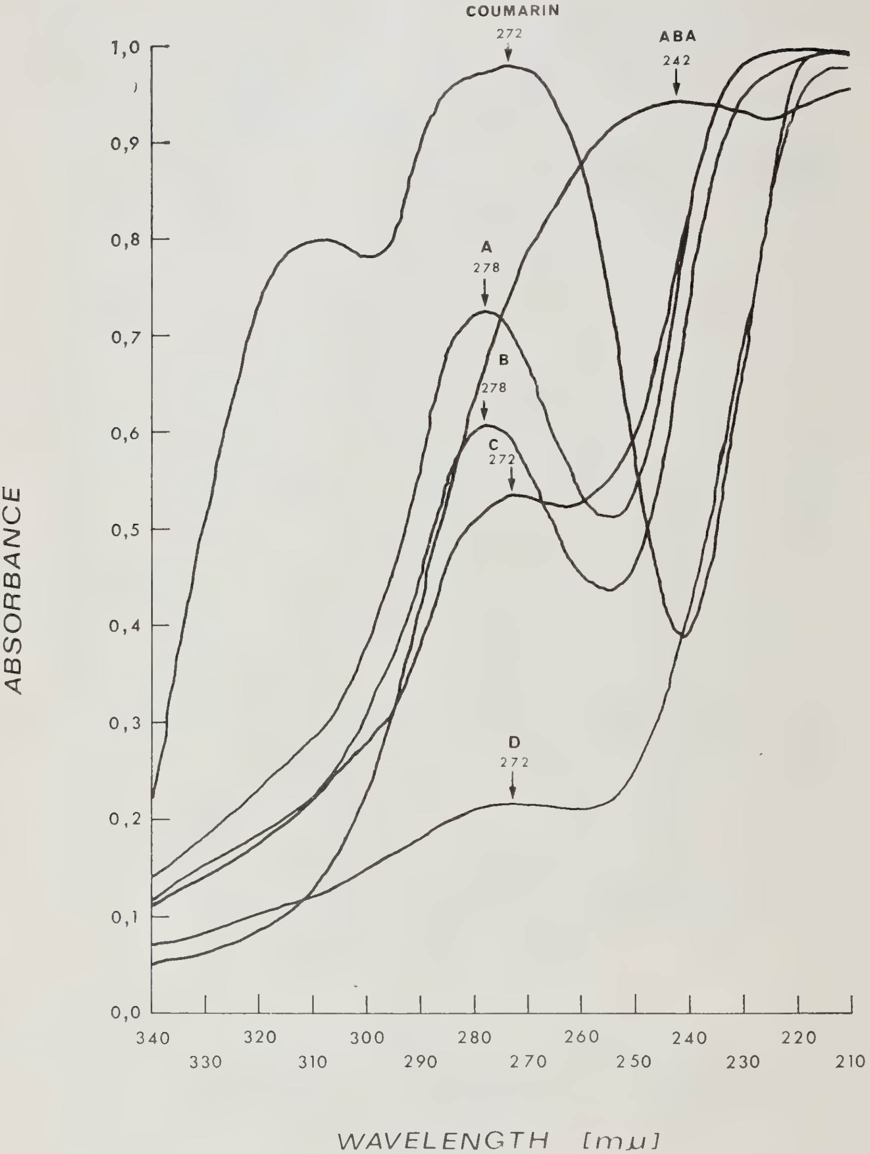
COUMARIN
272
ABA
242
A
278
B
278
C
272
D
272
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
ABSORBANCE
340
330
320
310
300
290
280
270
260
250
240
230
220
210
WAVELENGTH [mμ]

Fig 7.

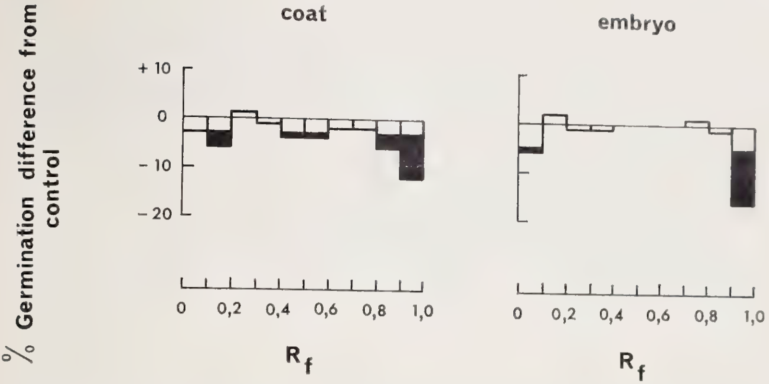

FIG. 8.
A comparison of the effect of ethyl acetate extracts of seed coat embryo of *Protea compacta* on germination of "Grand Rapids" lettuce seed in light. The values of the coat and embryo extracts are for the assay of 90 gms seed (6 × weight used in earlier assays). The solvent was *n*-butanol:ammonia:water. Shaded areas represent differences significant at 1% level.

ACKNOWLEDGEMENTS

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FIG. 7.
A comparison of the UV absorption spectra of ABA and coumarin and the ethyl acetate extracts of the seed coat and embryo of *Protea compacta*. The solvent was *n*-butanol:ammonia:water.
A = coat (R_f 0,5) B = coat (R_f 0,6) C = coat (R_f 1,0) D = embryo (R_f 1,0)

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